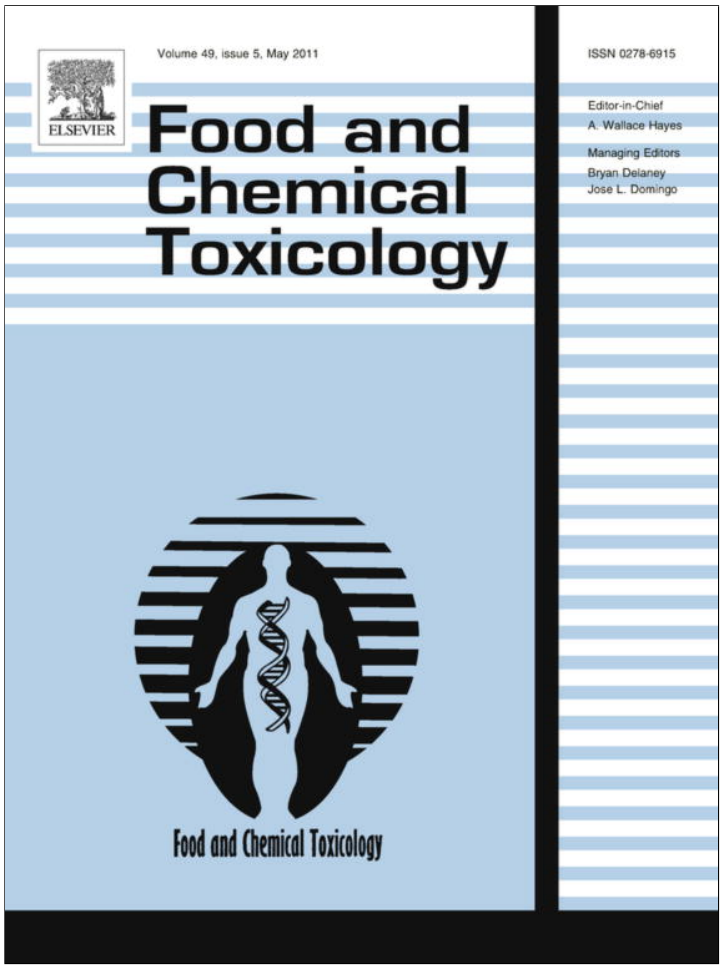

Food and Chemical Toxicology 49 (2011) 1063–1067

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Effects of chloroacetaldehyde in 2-chloroethanol-induced cardiotoxicity

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ARTICLE INFO

Article history:
Received 13 April 2010
Accepted 17 January 2011
Available online 23 January 2011

Keywords:
2-chloroethanol
Chloroacetaldehyde
Cardiotoxicity
nNOS
Nifedipine

ABSTRACT

Cardiovascular effects have often been found in 2-chloroethanol (2-CE) intoxicated patients, but the 2-CE elicits cardiovascular toxicity mechanism is not clear. Recently, we have found that chloroacetaldehyde (CAA) accumulation in 2-CE-intoxicated rat's blood and play an important role in 2-CE intoxication. In this study, we used an isolated rat atrium model to examine the cardiotoxicity of 2-CE and CAA. Results indicated that 2-CE did not cause tension arrest in isolated rat right atria, but CAA did. 2-CE caused tension inhibition in the isolated rat left atria. In addition, CAA caused significant tension inhibition and contracture in the isolated rat left atria. Nifedipine, an L-type calcium channel blocker, decreased CAA-induced tension inhibition and contracture. Meanwhile, atrial nNOS and calmodulin (CaM) had significantly greater expression in the 2-CE group and the CAA group than control group. Nifedipine could decrease CAA-induced nNOS and CaM expression. 2-CE-induced cardiovascular toxicity might be due to its metabolite CAA. CAA-induced cardiovascular toxicity might be mediated by calcium channel and nifedipine protected against nNOS-triggered cardiovascular effects.

1. Introduction

2-Chloroethanol (2-CE, ethylene chlorohydrin, CAS 107-07-3) is used as an intermediate product in the synthesis of ethylene oxide (Brashear et al., 1996), and the metabolite of halogenated hydrocarbons (Bhat et al., 1991). In Taiwan, some farmers apply 2-CE to grapevines to hasten sprouting. 2-CE poisonings and fatalities have been reported following oral exposure, inhalation or skin absorption. 2-CE exposure can result in severe metabolic acidosis, coma, respiratory failure, hypotension, and death after acute exposure (Wahlberg and Boman, 1978). Patients with these symptoms also present myocardial depression with shock, tachycardia, hypotension (Deng et al., 2001), and cardiac arrest (Miller et al., 1970). Chloroacetaldehyde (CAA) is formed via the metabolism of 2-CE by alcohol dehydrogenase in rat hepatocytes (Sood and O'Brien, 1993, 1994) and is also found in blood (Dubourg et al., 2001; Chen et al., 2010). Chloroacetaldehyde is the metabolite of side chain oxidation of ifosfamide (IF) made responsible for side effects (Bruggemann et al., 2006), IF have been associated with cardiotoxicity (Simbre et al., 2005), and CAA may play an important role in the cardiotoxic effects of IF (Loqueviel et al., 1997). It has been shown that CAA causes hypotension, respiration failure and induces cardiotoxicity in rabbits (Lawrence et al., 1972). Therefore, CAA has been demonstrated to be a cardiotoxic agent (Joqueviel et al., 1997), but the CAA induced cardiovascular toxicity mechanism is still unclear. Our previous results have shown that the vasorelaxation effects of 2-CE are mediated by CAA (Chen et al., 2009), and calcium channels might be involved in CAA-induced vasorelaxation. But little is known about how CAA-induced cardiovascular toxicity mechanism.

Nitric oxide synthase (NOS) has been found in neural structures relevant to cardiovascular control in the CNS and peripheral autonomic nervous system (Rodrigo et al., 1994; Egberongbe et al., 1994). Constitutive NOS has been found in vascular myocytes (Bernhardt et al., 1991; Boulanger et al., 1998) and cardiac myocytes (Kelly et al., 1996). Neuronal NOS (nNOS) has been suggested to target the cardiac sarcoplasmic reticulum (SR) (Xu et al., 1999) and regular heart tension. NO stimulation of SR Ca^{2+} release via the ryanodine receptor (RyR) and inhibited L-type calcium channel *in vitro* (Xu et al., 1998; Eu et al., 2000) suggests that nNOS has an opposite and facilitative effect on myocardial contractile reserve (Barouch et al., 2002). In this study, we used isolated left and right rat atria to examine the cardiotoxicity of 2-CE and CAA.

2. Methods

2.1. Chemicals

Nifedipine, 2-CE and CAA were purchased from Sigma Co. (St. Louis, MO, USA).

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doi:10.1016/j.fct.2011.01.013

2.2. Animals

Forty male Sprague–Dawley rats weighing 250–300 g were obtained from the BioLASCO Taiwan CO., Ltd. (I-Lan, Taiwan). Six groups for tension inhibition experiment (2-CE 1, 5, 10 mM; CAA 1, 5, 10 mM), one group for tension inhibition (nifedipine + CAA), and control group. Each group had five SD rats, and all rats' atria were collected for the immunohistochemistry assay after tension inhibition experiments. The animals were housed in cages with ample access to food (Lab Diet® 5001 Rodent diet, PMI® Nutrition International, LLC, St. Louis, MO, USA) and were provided with deionized reverse osmosis water *ad libitum*. The animals were kept at 20–25 °C and 40–70% relative humidity under a 12 h light on/off cycle. Experiments were performed according to the criteria for the use and care of experimental animals set out in "A Guidebook for the Care and Use of Laboratory Animals" (Yu et al., 2005). The study protocol was approved by the animal research ethics committee at National Chung Hsing University, Taichung, Taiwan (IACUC Approval No.: 98-42).

2.3. Preparation and treatment of the isolated rat atria

Rat atria were isolated according to our previous method (Hu et al., 2005), as described below. The right and left atria were separately isolated in normal Tyrode's solution (composition in mM: NaCl 94.8, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 7.78, and $CaCl_2$ 1.875; pH 7.4). The atria were cut into strips, suspended in a 10 ml organ bath and continuously exposed to 95% O_2 + 5% CO_2 at 37 °C. One end of the atria was fixed, while the other end was attached to a force displacement transducer. The spontaneous beating rate of the right atrium was recorded, and the left atrium was electrically stimulated with *S* rate: 0.1; delay time: 0; duration time: 0.5; and voltage: 70 (Grass SD9 stimulator, Quincy, MA, USA). The atria were equilibrated in Tyrode's solution and maintained with an optimal tension of 1 g for 60 min, with the solution being changed three times before use. Contractile tensions were recorded isometrically using a force displacement transducer connected to a Power-Lab/5 e recorder. Left atria preparations were treated with 2-CE (1, 5, 10 mM) or CAA (1, 5, 10 mM) and continuously recorded for 60 min for the response study. Another group was pre-treated with nifedipine (1 μM) for 10 min then treated with CAA (5 mM), and contractile tension was measured after incubation. For individual twitch, contractile force (*g*) was measured from the lowest tension (bottom) to the highest tension (top). The percentage of twitch inhibition was calculated as the decrease in tension (*g*) divided by the initial tension (*g*) and multiplied by 100. For contracture, the contracture force (*g*) was measured as the divergence between the baseline and maximal tensions. Right atria were prepared for spontaneous tension, treated with 2-CE (5 mM) or CAA (5 mM) and continuously recorded for 60 min.

2.4. Immunohistochemistry for nNOS in atria

Atria of rats were fixed with 10% neutral formalin solution after study for later use. The samples were embedded for immunohistochemistry stain with wax in 2 μm thick sections for light microscopic examination (Opticphot-2, Nikon, Tokyo, Japan). Atria tissue was harvested and dehydrated using xylene and alcohol. The samples were immersed in 10 M citrate buffer and heated to a boil for 20 min in a microwave oven. They were then processed for immunohistochemical staining using EnVision and Dual Link System Peroxidase (Cat. No. K5007, Dako Cytomation, Denmark). After reaction with 3% H_2O_2 for 10 min, the samples were marked using a Dako Pen (Red, CA, USA), immersed in PBS for 2 min and processed with primary antibodies against nNOS/NOS type 1 (1:500, BD, Franklin Lakes, NJ USA) or calmodulin (CaM) (1:1000, Calbiochem, Darmstadt, Germany) for 2 h. Next, the samples were washed with PBS, incubated with peroxidase-labeled polymer-HRP, again washed with PBS, and treated with chromogen and finally stained with Mayer's Hematoxylin. The number of nNOS-positive cells in smooth muscle was detected under microscopy at 200× magnifications and calculated using the Image Pro-Plus image system (Image Pro-Plus, CA, USA).

2.5. Statistical analysis

The half maximal inhibitory concentration (IC_{50}) was determined with 95% confidence limits. The means and standard deviations (SD) of the experimental data were also computed for each group. Treatments showing significant differences were further analyzed using the paired Student's *t-test*. A *P* value of below 0.05 was considered to indicate statistical significance, as indicated by an asterisk.

3. Results

3.1. Effects of 2-CE and CAA in isolated rat right atria

In the isolated right atrial intercaval region containing the sinoatrial node (SA node) (Oosthoek et al., 1993), there was spontaneous tension. 2-CE caused tension inhibition but did not cause tension arrest in isolated rat right atria until 60 min (Fig. 1). CAA induced tension inhibition in isolated right atria and caused tension to arrest within 23.4 ± 1.2 min (Fig. 1).

3.2. Effects of 2-CE and CAA in isolated rat left atria

In this study, we found that 2-CE (1, 5, 10 mM) caused tension inhibition in isolated rat left atria of 8.0, 21.8 and 33.8%, respectively (Table 1. IC_{50} > 10 mM, *: $P < 0.05$ versus control). CAA (1, 5, 10 mM) caused significant tension inhibition of 19.4, 44.5 and 68.2%, respectively (Table 1. IC_{50} = 6.7 mM, *: $P < 0.05$ versus control, $^{\#}$: $P < 0.05$ versus 2-CE). Moreover, CAA also induced contracture of left atria (Fig. 2).

3.3. Effects of nifedipine on CAA-induced tension inhibition and contracture

We used the L-type calcium channel blocker nifedipine (Wang et al., 2005) to examine the effects of CAA in cardiotoxicity. Atria pre-treated with nifedipine exhibited significantly decreased tension inhibition (32.7 ± 8.1% versus CAA) and decreased contracture in response to CAA (Fig. 2. *: $P < 0.05$ versus CAA).

3.4. nNOS and CaM expression in 2-CE, CAA and nifedipine pre-treated atria

We examined atria for nNOS and CaM expression by IHC to measure direct effects of cardiotoxicity. The results indicated that the area of nNOS expression in the control group atria was 5.6% of total area (Fig. 3). On the other hand, the area of nNOS expression in samples incubated with 2-CE (5 mM), CAA (5 mM), or pre-treated with nifedipine (1 μM) and then exposed to CAA (5 mM) were 45.8% ($^{*}P < 0.05$ versus control), 60.8% ($^{*}P < 0.05$ versus control) and 8.5% ($^{\#}P < 0.05$ versus CAA), respectively (Fig. 3). Meanwhile, CaM expression in control group rat atria had an area of 22.4% of total area (Fig. 4), while CaM expression in samples incubated with 2-CE (5 mM), CAA (5 mM), or pre-treated with nifedipine (1 μM) and then exposed to CAA (5 mM) was 64.1% ($^{*}P < 0.05$ versus control), 54% ($^{*}P < 0.05$ versus control) and 10.6% ($^{\#}P < 0.05$ versus CAA), respectively (Fig. 4). These results indicate that CAA caused tension inhibition and contracture in left atria that were mediated by calcium channels.

4. Discussion

2-CE intoxication has been reported to cause myocardial depression with shock (Deng et al., 2001), or cardiac arrest (Miller et al., 1970) cardiovascular toxicity in humans and vasorelaxation in isolated rats' aortic rings (Chen et al., 2009). Our results show that 2-CE did not cause tension arrest in rat right atria within 60 min, but CAA cause tension arrest within 23.4 ± 1.2 min. It has been showed that CAA induced cardiac arrest in less than 90 min in rabbit (Lawrence et al., 1972). Miller et al. reported that 2-CE-intoxicated patient developed profound hypotension, tachycardia, cardiac arrest, and died (Miller et al., 1970). This result indicating that the CAA may cause 2-CE-induced cardiotoxicity and fatality.

Our results show the IC_{50} of CAA in atria tension inhibition was 6.7 mM, but the IC_{50} of 2-CE was more than 10 mM, which suggests that 2-CE-induced cardiotoxicity is mediated by its metabolite CAA. Ifosfamide (IF) undergoes considerable chloroethyl side-chain oxidation with liberation of CAA (Bruggemann et al., 2006). High doses of IF are associated with severe but usually reversible cardiotoxicity (Quezado et al., 1993). The concentration of CAA in the blood of patients receiving IF treatment can be as high as 100 μmol/l (Goren et al., 1986), and can be concurrent liberated

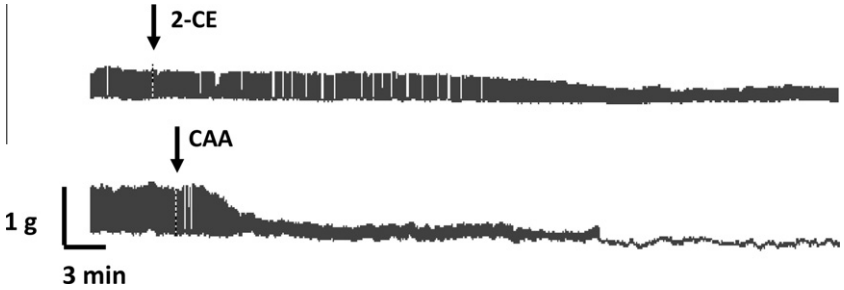

Fig. 1. Effects of 2-Chloroethanol (2-CE) and chloroacetaldehyde (CAA) on the twitch tensions of isolated rat right atria. 2-CE or CAA at concentrations of 5 mM were individually added to right atria and continuously recorded for 60 min.

Table 1
Tension inhibition of isolated rat left atria by 2-CE and CAA.

Conc. (mM)	Tension Inhibition (%)	
	2-CE	CAA
1	8.0 ± 5.0	19.4 ± 10.2*
5	21.8 ± 12.7*	44.5 ± 5.7*,**
10	33.8 ± 6.3*	68.2 ± 19.8*,**

IC_{50}: 2-CE was higher than 10 mM and CAA was 6.7 mM.
* $P < 0.05$ versus control.
** $P < 0.05$ CAA versus 2-CE ($n = 5$).

20% CAA to cause cardiotoxicity (Wanger, 1994). It has been shown that CAA i.v. injected into rabbits at a dosage around 20 mg/kg/bw produces lethal cardiotoxicity (Lawrence et al., 1972). In our result 5 mM of CAA caused cardiac arrest in isolated rat right atria within 23 min. In our knowledge, little was known about CAA-induced cardiotoxicity mechanism. Previous reports indicated that 2-CE-intoxicated patients showed the cardiotoxicity symptoms as hypotension, cardiac arrest, and tachycardia (Miller et al., 1970; Deng et al., 2001); CAA-intoxicated rabbits showed the cardiac arrest within 90 min (Lawrence et al., 1972); 2-CE induced severe intoxication and death within 1 h in rats (Chen et al., 2010). Our result showing that 2-CE metabolite, CAA caused cardiotoxicity as cardiac arrest and contracture by using isolated rat atria, but not 2-CE.

It has been reported that NOS inhibition induced an increase in peripheral arterial resistance and arterial elastance, with a concomitant advance reduction of cardiac output in acute ischemic heart failure (Nordhaug et al., 2004). Our results show that 2-CE and CAA caused a significant increase in nNOS and CaM protein expression in isolated rat atria, which indicates that nNOS expression may be involved in 2-CE- and CAA-induced acute heart failure. It has been found that inhibition of nNOS prevents the cholinergic inhibition of the L-type Ca^{2+} current in adrenergically prestimulated SA node cells (Han et al., 1994), and that nNOS plays a necessary role in the autonomic control of heart rate. nNOS activity is primarily regulated by increases in intracellular Ca^{2+}, which activate nNOS through CaM binding (Bredt and Snyder, 1990). nNOS is reversibly regulated by calcium CaM or alternatively possess a tightly bound CaM subunit; therefore CaM might bind and activate nNOS (Marletta, 1993, 1994; Masters, 1994). Our results show that pre-treatment with nifedipine decreased CAA-induced nNOS and CaM expression in isolated rat atria. These results indicate that CAA-induced cardiovascular toxicity might be triggered by nNOS and calcium channel might be involved.

In our previous study, i.p. injection of 2-CE 12 mg/kg formatted 48.8 μg/L CAA in SD rat's blood and caused rat died within 1 h (Chen et al., 2010). It was hard to observe the cardiotoxicity symptoms by using lethal examination. In this study, we using *in vitro* isolated rat's left and right atria to examine the 2-CE fatal intoxication were associated with acute cardiotoxicity. Calcium signaling inhibitor, nifedipine attenuated the initial NO elevation (Liao et al., 2006), also in our previous result showed that nifedipine could decrease CAA-induced vasorelaxation (Chen et al., 2009). Nifedipine is used to treat high blood pressure and to control angina. It works by relaxing the blood vessels so the heart does not have to pump as hard. It also increases the supply of blood and oxygen to the heart.

2-Chloroethanol is switched to CAA via alcohol dehydrogenase which is then detoxified by conjugation with glutathione (GSH) and liberation of hydrogen chloride (Deng et al., 2001; Chen et al., 2010). The toxicological mechanisms of 2-CE remain unclear, it might be due to intracellular formation of CAA (Miller et al., 1970; Bhat et al., 1991; Chen et al., 2010). Chloroacetic acid, a metabolite of CAA, can further inhibit the tricarboxylic acid cycle enzymes and bind to GSH or other sulfhydryl-containing substances (Miller et al., 1970; Deng et al., 2001), and cause severe tissue damage in energy rich organs such as the heart, central nervous system, and skeletal muscles, and manifest as malaise, vomiting, myocardial depression (Deng et al., 2001). Our previously results indicated that combined with fomepizole and *N*-acetylcysteine could decrease blood CAA formation and detoxify

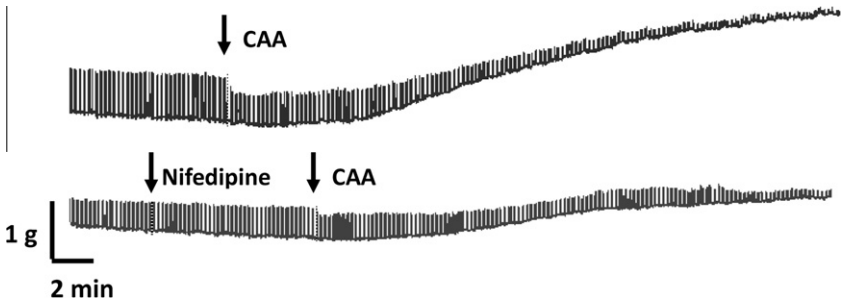

Fig. 2. Effects of chloroacetaldehyde (CAA) on the twitch tensions of isolated rat left atria. CAA at concentrations of 5 mM or pre-treated with nifedipine (1 μM) then CAA were individually added to left atria and continuously recorded for 60 min.

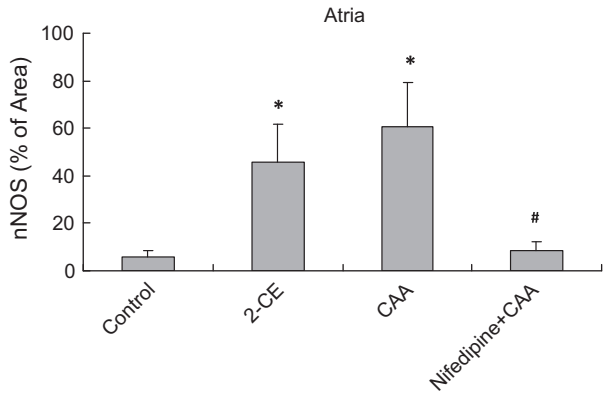

Fig. 3. nNOS IHC results in rat isolated atria (*$P < 0.05$ versus control, #$P < 0.05$ versus CAA alone).

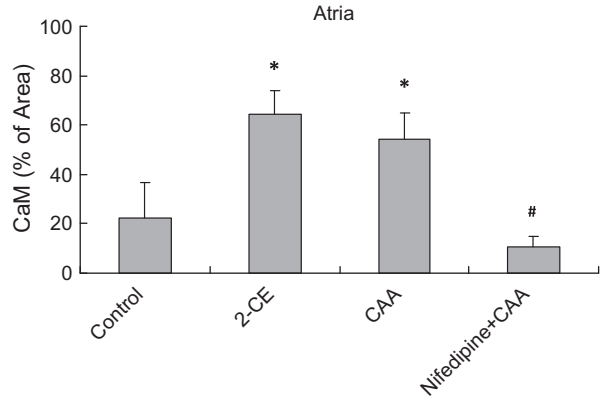

Fig. 4. CaM IHC results in rat isolated atria (*$P < 0.05$ versus control, #$P < 0.05$ versus CAA alone).

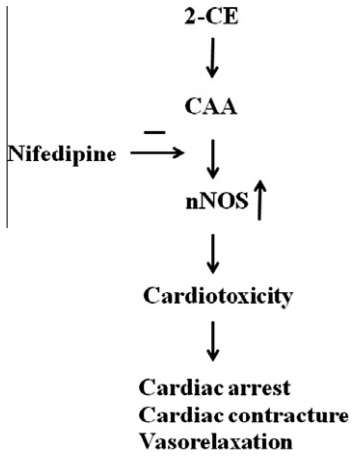

Fig. 5. The proposed mechanisms of 2-CE and CAA induced cardiotoxicity. 2-CE metabolite, CAA induced atria nNOS overexpression and caused cardiotoxicity of cardiac arrest, cardiac contracture, and vasorelaxation. Nifedipine, a calcium channel blocker could decrease nNOS overexpression and decrease CAA-induced cardiotoxicity.

of 2-CE-intoxicated rat (Chen et al., 2010). The strategy for 2-CE-intoxicated patients' therapy may provide fomepizole and *N*-acetylcysteine to prevent and decrease the CAA formation first, and provide the calcium channel blocker nifedipine to decrease the heart damages. But the potential antidotes for 2-CE-intoxicated treatment need further examination.

In conclusion, 2-CE caused cardiovascular effects as cardiac arrest, cardiac contracture, and vasorelaxation; it may be due to its metabolite, CAA (Fig. 5), which is triggered by atria nNOS overexpression in rat. L-type calcium channel blockers, nifedipine might be protective against CAA-induced cardiovascular toxicity.

Conflict of Interest

None

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